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Hemocyanin Respiratory Pigment in Bivalve Mollusks

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Hemocyanins, high molecular weight oxygen-binding proteins, were identified in two species of protobranch bivalve mollusks, *Acila castrensis* and *Yoldia limatula*. Although hemocyanins have been reported in chitons, gastropods, and cephalopods, they have not been observed in the Class Bivalvia. In *A. castrensis* the dissociation products of hemocyanin, characterized by gel electrophoresis, had a subunit molecular weight of approximately 250K. Negatively stained preparations of extracted hemocyanin formed protein aggregates in the shape of cylinders measuring 35 by 38 nanometers. X-ray microanalysis of hemocyanin aggregates in thin sections of *Y. limatula* demonstrated the presence of copper in the molecules. The discovery of hemocyanin in the protobranchs reinforces the primitive nature of the taxon and is further evidence that the major molluscan classes have a common ancestry.

HEMOCYANINS ARE HIGH MOLECULAR weight, copper-containing pigments in the hemolymph of mollusks (*1–4*) and arthropods (*5*). In mollusks, hemocyanins have been found in chitons, gastropods, and cephalopods but to our knowledge have not been reported in bivalves. However, while investigating the ultrastructure of protobranch pericardial glands (*6*), we noted that the hemolymph contained structures that resembled characteristic molluscan hemocyanin molecules (*7*). Further studies were undertaken to characterize the respiratory protein in two protobranch species, *Acila castrensis* (Hinds) and *Yoldia limatula* (Say). We examined the molecular weight, size, and shape of the aggregates and tested for the presence of copper in the protein.

Preparations enriched in the respiratory pigment were obtained from *A. castrensis* and *Y. limatula* by a modification of the procedure of Waxman (*8*). Pellets from the final centrifugation were resuspended in collecting fluid and examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*9*). Polypeptides of *A. castrensis* hemocyanin comigrated with those of the hemocyanin of the limpet, *Megathura crenulata*, at an estimated molecular weight of 250K (Fig. 1). The *Y. limatula* preparation had two prominent polypeptides of approximately 250K and 200K, the latter possibly being a breakdown product of hemocyanin or of an unrelated polypeptide (Fig. 1).

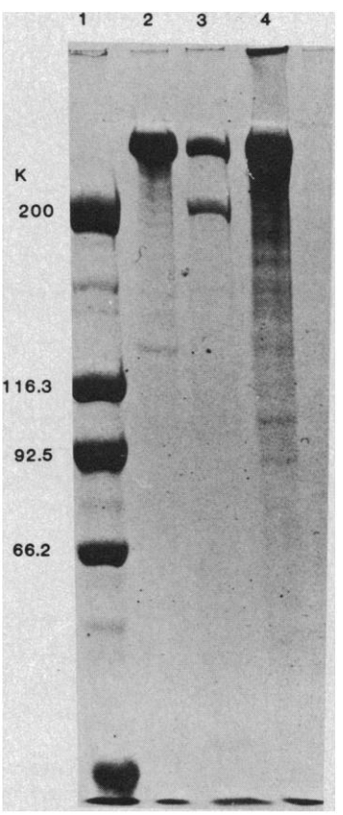

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of hemocyanin from *A. castrensis* (lane 2) and *Y. limatula* (lane 3). For comparison, limpet hemocyanin (Sigma) is shown in lane 4. Lane 1 contains molecular weight markers: myosin (200K), β-galactosidase (116K), phosphorylase B (92.5K), bovine serum albumin (66K), and ovalbumin (45K) (Pharmacia).

Samples of the hemocyanin-enriched preparations of *A. castrensis* and *Y. limatula* used to determine molecular weights also were negatively stained and viewed with a transmission electron microscope. The hemocyanin of *A. castrensis* (Fig. 2A) showed the characteristic cylindrical configuration of the macromolecular aggregates, and each aggregate was made up of six tiers. Hemocyanin of the heart hemolymph of *A. castrensis* (Fig. 2B) also showed the characteristic shape of the protein. The cylinders appeared circular in end view and rectangular in side view; they averaged 35 nm in height and 38 nm in width ($n = 5$). The negatively stained hemocyanin from the preparation extracted from *Y. limatula* was not as well defined as that from *A. castrensis*; however, the cylindrical configuration of the molecular aggregate was again evident (Fig. 3C).

Thin sections of the auricle of *Y. limatula* (Fig. 3A) were used for x-ray microanalysis (*10*) to establish the presence of copper in the aggregates. The microanalysis results (Fig. 3B) indicate the presence of significant energy peaks of both the K- and L-shell electrons of copper. Analysis from nearby nonhemal tissues revealed no significant peaks for copper. Furthermore, during the procedures to isolate hemocyanin from *A. castrensis* (*8*), it was observed that the hemocyanin pellet was bright blue. As noted by Mangum (*3*), oxygenated hemocyanin molecules appear blue because of the presence of copper atoms.

The uniqueness of hemocyanins in arthropods and mollusks has led to numerous investigations into the structure and function of the proteins and speculation as to their evolutionary importance in mollusks. Investigators have searched for hemocyanins

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in bivalves (*2, 11*), but the protobranchs seem to have been overlooked. Bivalves that have been surveyed either lack a respiratory pigment or have globins. The globins may be circulating in the hemolymph (hemoglobins) or within muscle and nerve cells (myoglobins). Of respiratory pigments investigated, the shape of the extracellular hemoglobin described in the hemolymph of *Cardita borealis* (elongated cylinders or rods of various lengths) (*12*) has some similarity to that of the protobranch hemocyanin. However, in the protobranchs the aggregates contain copper, which is lacking in the hemocyanin of *C. borealis*. The protobranch hemoglobin aggregates closely resemble those of gastropods in macromolecular size and shape (*2*). The constituent polypeptides in bivalves appear to have a lower subunit molecular weight (250K) than in gastropods. It should be noted, however, that molecular weight estimates in this range cannot be made very accurately.

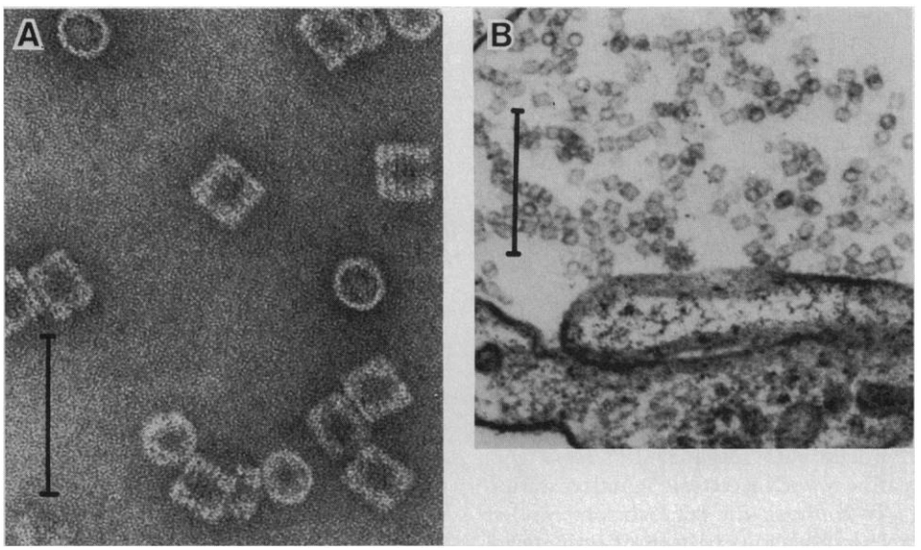

Fig. 2. *Acila castrensis*. (A) Electron micrograph of hemocyanin aggregates. A sample of the SDS-PAGE preparation (*9*) was placed on a Formvar-coated grid and negatively stained with 1 percent uranyl acetate. Scale bar, 100 nm. (B) Electron micrograph of a section of the auricle showing hemocyanin molecules in the hemolymph. The tissue was fixed in 4 percent glutaraldehyde in 0.2*M* cacodylate buffer, with NaCl and sucrose added for an osmolality of 1.3 osmol/kg at a *p*H of 7.5, and postfixed in 1 percent osmium in 0.2*M* cacodylate buffer (*p*H 7.2). Scale bar, 300 nm.

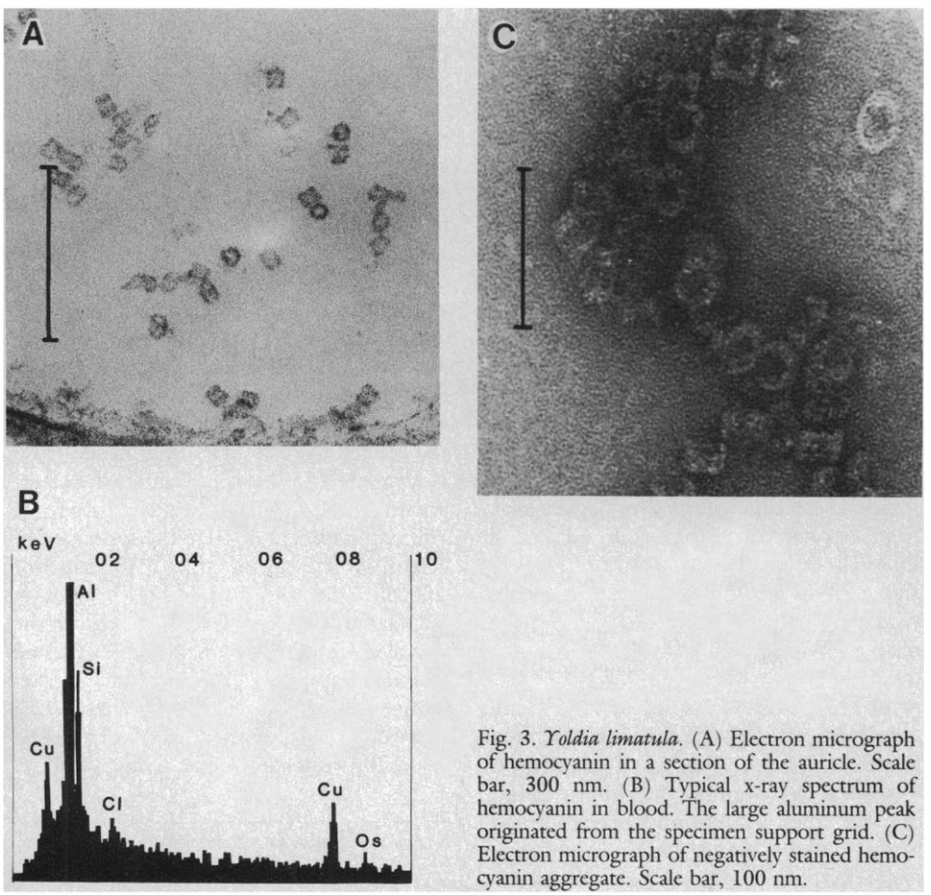

Fig. 3. *Yoldia limatula*. (A) Electron micrograph of hemocyanin in a section of the auricle. Scale bar, 300 nm. (B) Typical x-ray spectrum of hemocyanin in blood. The large aluminum peak originated from the specimen support grid. (C) Electron micrograph of negatively stained hemocyanin aggregate. Scale bar, 100 nm.

Most protobranch bivalves are small (less than 3 cm), live in mud habitats, have simple paired respiratory ctenidia, and are deposit-feeders (*13*). Unlike more advanced bivalves, the protobranchs have simple gills with nonreflected filaments which only function for respiration; they do not have siphons to aid in water exchange within the mantle cavity. With this primitive mantle cavity design, it is not surprising to find a respiratory pigment of primitive occurrence with reference to its wide distribution within basic molluscan groups. It is here undoubtedly associated with oxygen transport. Among the highly evolved bivalves most species, large and small, have become functionally adapted for filter feeding; this is manifested in an increase in the number and size of gill filaments, enlargement of the mantle cavity, development of increased and directed water flow facilitated by muscular siphons, and structural modifications of the gill cilia and tissues to make the gill an efficient feeding organ. In these filter-feeding bivalves the globins may have become adapted for specialized functions.

The presence of hemocyanin in protobranchs is further evidence that hemocyanin was the primitive oxygen carrier in mollusks and therefore may well have been associated with premolluscan ancestors. This occurrence compounds the problems that remain to be addressed in the structure and function of the hemocyanins in other mollusks and in arthropods.

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several times through the viscera and foot; the hemolymph, after being strained through 250-μm nylon mesh, was added to an approximately equal volume of collection fluid consisting of 0.5*M* NaCl, 0.05*M* Hepes, 5 m*M* EGTA, 1 m*M* *p*-tosyl arginine methyl ester, Pepstatin A (3 μg/ml, Boehringer-Mannheim), and leupeptin (3 μg/ml, Boehringer-Mannheim) (*p*H 7.5). After the hemolymph preparation was centrifuged at 10,400*g* in a Sorvall centrifuge with an HB-4 rotor, fluid between the pellet and the floating lipid pad was removed and recentrifuged. The supernatant was then spun for 2 hours at 80,000*g* in an SW-39 rotor with an L-2 Beckman ultracentrifuge; the resulting pellet was used for gel electrophoresis.

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Flying Primates? Megabats Have the Advanced Pathway from Eye to Midbrain

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The pattern of connections between the retina and midbrain has been determined with electrophysiological and neuroanatomical methods in bats representing the two major subdivisions of the *Chiroptera*. Megachiropteran fruit bats (megabats), *Pteropus* spp., were found to have an advanced retinotectal pathway with a vertical hemidecussation of the kind previously found only in primates. In contrast, the microchiropteran bat *Macroderma gigas* has the "ancestral" or symplesiomorphous pattern of retinotectal connections so far found in all vertebrates except primates. In addition to linking primates and megachiropteran bats, these findings suggest that flight may have evolved twice among the mammals.

THE PATTERN OF CONNECTIONS BETWEEN the retina and the midbrain superior colliculus (or tectum) distinguishes primates from all other mammals so far studied (*1*). In strepsirhine and haplorhine primates (*2*), the pattern of crossover of retinotectal fibers is like that of the retinothalamic fibers, with the result that the superior colliculus on one side of the brain subserves both eyes but only the opposite hemifield of visual space (*3*). In contrast, in all other vertebrate groups so far examined, the crossover pattern of retinotectal fibers differs from that of the retinothalamic fibers, with the result that the superior colliculus subserves the whole visual field of the opposite eye (*4–10*). Bats have not previously been studied in this regard, although the question is of some interest because some scholars have placed bats together with primates, dermopterans, and tree shrews in the superorder Archonta (*11*). Moreover, there are two distinct bat assemblages, one of which has an advanced visual organization with similarities to that of the primates. The latter are megabats, suborder *Megachiroptera*, a uniform Old World group of large, vegetarian bats reliant on their highly developed vision for foraging and obstacle avoidance (*12*). The microbats, suborder *Microchiroptera*, are, by contrast, diverse, worldwide, small, predominantly insectivorous bats, all of which use ultrasonic emissions for echolocation (*13*). I now report that fruit bats of the genus *Pteropus* have the advanced pattern of retinotectal fiber connections like that of primates. In contrast, the microbat, *Macroderma gigas*, has the plesiomorphous pattern of retinotectal projection found in most vertebrates. The findings lend support to the older classifications linking primates and bats, with the new qualification that this applies only to the *Megachiroptera*. A corollary of this phylogenetic hypothesis is that mammalian flight has evolved independently more than once (*14*).

The megabats used in this study were three grey-headed flying foxes, *Pteropus poliocephalus*, two black flying foxes, *Pteropus alecto*, and one little red flying fox, *Pteropus scapulatus*, taken from the wild near Brisbane, Australia, and maintained in an outdoor aviary. The microbats were two Australian ghost bats, *Macroderma gigas*, taken from a colony of 400 breeding females at Pine Creek, Northern Territory. *Macroderma* was chosen because, in comparison with most other microchiropterans, it has a relatively well-developed and experimentally tractable visual system, with large eyes and a temporal retinal area of increased ganglion cell density which "looks" forward like that found in *Pteropus* (Fig. 1). Two methods were used to determine the pattern of retinotectal fiber connections: electrophysiological and neuroanatomical. In the first, microelectrodes were used to record the visual responses of individual neurons in the superior colliculus of bats anesthetized with intramuscular injections of ketamine and xylazine (*15*). The locations of visual receptive fields were plotted with respect to the zero vertical meridian for each eye, the latter having been established from the projection of the ophthalmoscopically visible optic nerve head and data from retinal whole mounts giving the relation between the area of increased ganglion cell density and the nerve head (*16*). A check on this estimate was provided by binocular fields recorded in overlying visual cortex (*17*). For the neuroanatomical studies, injections of horseradish peroxidase (HRP), in some cases conjugated to wheat germ agglutinin (WGA-HRP), were made into the superficial layers of the superior colliculus receiving retinal input to enable retrograde labeling of retinal ganglion cells (*18*). The previous electrophysiological determination of the location of the superior colliculus was used to guide the placement of the HRP injections in three cases. In five other cases, the overlying visual cortex was removed by suction ablation to expose the superior colliculus so that injections could be directed visually to its whole retinal projection area. After survival times of 24 to 72 hours, the animals were killed with an overdose of anesthetic, perfused with fixative, and the brain and eyes removed. Retinal whole mounts were prepared to show the pattern of labeled retinotectal ganglion cells and the brain sectioned and reacted to verify the injection site (*19*).

All three *Pteropus* spp. examined electrophysiologically had the primate pattern of retinotopic organization in the superior colliculus. At the caudal edge of the superior colliculus, neurons had receptive fields in the far contralateral field, whereas at the rostral edge receptive fields were located close to the zero vertical meridian. No receptive fields were found more than 5° (the accuracy inherent in the method of plotting landmarks) into the ipsilateral hemifield. A

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